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Characterization of Monoclonal Antibodies Raised against Rat and Chicken Hepatic Actinogelins and Chicken Gizzard α-actinin

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SUMMARY

Twenty-five monoclonal antibodies raised against rat and chicken hepatic actinogelins and chicken gizzard α-actinin were surveyed for their affinity toward cytoskeletal proteins. Most of the antibodies showed different reactivity toward native and denatured antigens. Some antibodies cross-reactied with four α-actinin family proteins, *i. e.* rat and chicken hepatic actinogelins and rat skeletal muscle and chicken gizzard α-actinins. Other groups of antibodies cross-reacted with proteins belong to α-actinin family and some other cytoskeletal proteins.

A monoclonal antibody designated "A-I" which is cross-reactive with α-actinin family proteins and filamin were studied further for its reactivity to proteolytic fragment of cytoskeletal proteins. Since this antiboby is reactive with a specific domain of the cross-reactive proteins, a hypothesis that highly conserved (possibly actin-binding) domain may be present in these proteins obtained some experimental support.

Key words: Dot-immunobinding, Peptide maps, Immunoblotting, Denaturation, Cross-reactivity

INTRODUCTION

Advantages of using monoclonal antibodies seems to be as follows: 1. they can detect very small differences in protien structure, such as difference of only one amino acyl residue; 2. antibody reacting with a single epitope can be raised using

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[1] Abbreviations used are: SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline

crude antigens; 3. they can be used as a probe for detection of a small domain in different proteins. On the other hand, monoclonal antibodies have some drawbacks, such as that they react similary with quite different proteins, if they contain the same or homologous epitopes (probably consisting of about five amino acyl residues) (14).

Cytoskeletal proteins, such as components of the microfilament system, have some common characteristics. Since they have many different sites functioning for binding with other components, their structure is very highly conserved (12, 13). Moreover, a possibility was considered that a common domain for binding to certain site(s) of actin is present in different actin-binding proteins, since some proteins bind to the same or an overlapping site on the actin molecule (8). Monoclonal antibodies may be useful for exploring such a possibility.

Actinogelin is a Ca^{2+}-regulated actin cross-linking protein which was discovered by us (7) and is present in a wide variety of animal cells (6). This protein seems to have some similarity with a muscle protein, α-actinin, in their molecular mass, molecular shape and actin-binding site (6, 9), although Ca^{2+}-sensititvity and gelation activity are quite different (9). Therefore, we have attempted to prepare monoclonal antibodies raised against actinogelins and α-actinin. Characterization of the monoclonal antibodies and a survey of epitope in α-actinin family proteins will be reported.

EXPERIMENTAL PROCEDURES

Purification of proteins

Rat hepatic actinogelin and chicken gizzard α-actinin were prepared as described previously (6). Chicken hepatic actinogelin was purified by a method similar to that used for its isolation from rat liver (3, 6). The outline of the purification procedure is as follows: Chicken liver extract was subjected to ammonium sulfate fractionation, and pellets obtained between 15-35% saturation were dialyzed, and chromatographed on a DEAE-Sephacel column. Fractions eluted with 0.35M KCl were collected, and applied onto a hydroxylapatite column. Elution was performed by a linear gradient of 50-200 mM potassium phosphate buffer, pH6.8. Fractions containing actinogelin were gel-filtrated with a Sepharose 6B column. Further purification was achieved by rechromatography on DEAE-Sephacel. A linear gradient of KCl was applied onto the column, and an eluted fraction(s) which was relatively pure, was used as the final preparation. From rat liver or Ehrlich tumor eclls, pure actinogelin is readily obtained at this step (3, 7). As judged from SDS[1]-gel electrophoretic pattern, however, the final prepartion was still contaminated with several minor components, some (or most) of which may be proteolytic fragments of the native protein, since many of these

minor components also possessed actin binding ability (data not shown).

Actin, filamin, vinculin, tropomyosin and heavy meromyosin were purified as described previously (5, 6, 8).

Preparation of monoclonal antibodies

Monoclonal antibodies against rat hepatic antinogelin were prepared as described previously (5) using Balb/c mice and myeloma cells [P3/×63−Ag8. U1 (P3−U1)]. Antibodies against chicken gizzard α-actinin were prepared in a similar manner. Since the actinogelin prepared from chicken liver was not sufficiently pure for its direct use as an antigen, SDS-denatured pure actinogelin obtained on gel electrophoresis was used as an antigen. For immunization, each mouse recieved four i. p. injections, each containing 30-50 μg of actinogelin in 0.25-0.3m*l* finely divided gel suspension in PBS, pH7.4, at one week intervals. Spleen cells from mouse showing high titres (1/40,960 as determined by avidin-biotin system) were used for making hybridoma. The fusion procedure was essentially the same as that for actinogelin except that the spleen cell to myeloma cell ratio was 60 : 1. Ascites fluid containing monoclonal antibodies was obtained as described previously (5).

Dot immunobinding assay

Dot immunobinding assay for native (undenatured) proteins was performed as described previously (5), except that native chicken hepatic actinogelin was used when indicated. The assay for denatured antigens was done by using antigens treated at 100℃ for 3 min in a medium consisting of 30 mM Tris-HCl buffer, pH 6.8, 2.5% mercaptoethanol and 1% SDS. Other conditions were the same as those described for native antigens.

Immunoblotting, SDS-gel electrophoresis and quantification of protein

These were carried out as described previously (5).

Proteolytic cleavage of proteins

One dimensional peptide mapping of denatured proteins using V8 protease was performed as described previously (5).

Cleavage of native proteins was carried out as follows: native proteins (6 μg) were digested with 0.12μg of V8 protease, chymotrypsin or trypsin at 37℃ for 35 min in a medium (50 μ*l*) consisting of 0.5% ammonium bicarbonate and 0.1mM $CaCl_2$.

RESULTS

Characterization of Monoclonal Antibodies by Dot Immunobinding Assay

Monoclonal antibodies were prepared by a conventional method (5) against three related proteins, including rat hepatic actinogelin, chicken gizzard α-actinin and chicken hepatic actinogelin. Native (intact) proteins were used for immunization except for chicken hepatic actinogelin which was denatured by heating in 1% SDS. Among approximately 100 hybridoma clones isolated, 25 clones (15 for chicken hepatic actinogelin (chA), 5 for rat hepatic actinogelin (A) and 5 for chicken gizzard α-actinin (chα or α)) were selected and propagated in mouse abdominal cavity.

Monoclonal antibodies produced by these clones were subjected to binding specificity test using native antigens. As shown in Fig. 1, antibodies exhibited quite different specificities against several cytoskeletal proteins, including rat actinogelin, rat skeletal muscle α-actinin, chicken gizzard α-actinin, chicken filamin, chicken vinculin, rabbit heavy meromyosin, rabbit skeletal muscle actin, and rabbit skeletal muscle tropomyosin. Most of the antibodies against denatured actinogelin have a broad specificity and a rather low affinity toward native antigens. All of the antibodies against native actinogelin or α-actinin cross-reacted with three α-actinin family proteins, *i. e.* rat actinogelin, rat skeletal muscle α-actinin and chicken gizzard α-actinin. Some of them also reacted with chicken gizzard filamin and bovine serum albumin.

Binding ability of the antibodies to denatured antigens was also surveyed (Fig. 2). Some of the antibodies prepared against denatured actinogelin, such as chA-I, chA-VI and chA-VII, reacted with all proteins tested. Another group of the antibodies, such as chA-III, ChA-IV, chA-IX, chA-X, chA-XII and chA-XIII, reacted with intact chicken gizzard α-actinin weakly, but only marginally, if any, with the other antigens. Antibodies which reacted strongly with the original immunogen, *i. e.* denatured chicken hepatic actinogelin, were chA-I, chA-II, chA-VI, chA-VII and chA-XIV.

All of the antibodies reacted differently with native and denatured antigens. About half of the antibodies prepared against native antigens, *i. e.* rat hepatic actinogelin and chicken gizzard α-actinin, were not cross-reacted with denatured antigens. Some of them, such as α-I, α-II, A-I, A-II and A-IV, reacted with denatured chicken actinogelin to some extent.

Specificity of Binding of the A-I Antibody to Proteolytic Fragments of Several Antigens

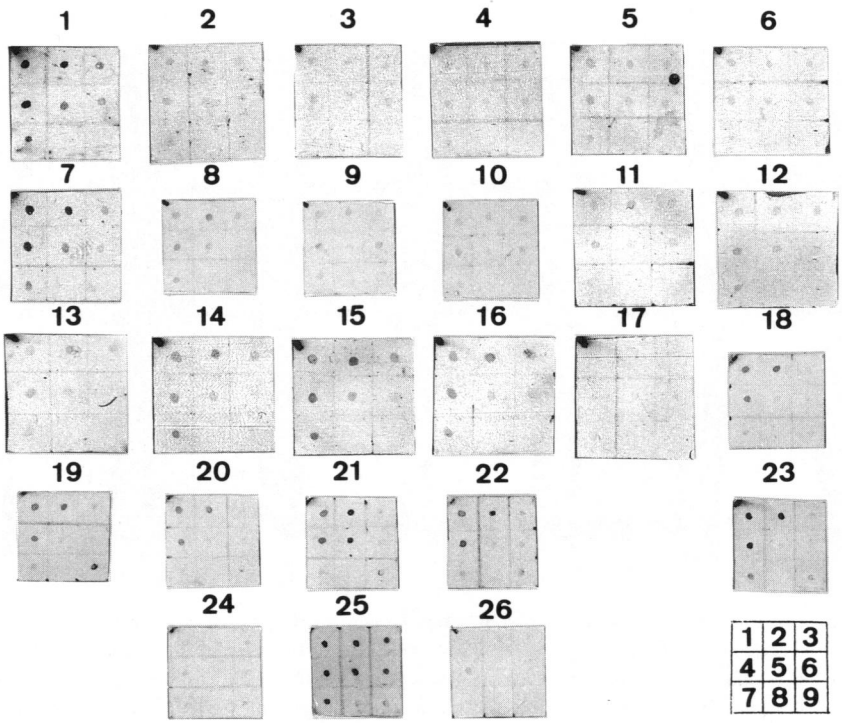

Fig. 1 Dot immunobinding assay of monoclonal antibodies using native antigens.
For each antibody, binding ability to nine different proteins was surveyed. As shown at the lower left corner of the figure, these proteins were spotted (0.2 μl of 0.5mg/ml solution) on nitrocellulose paper in a regular pattern as follows: 1, rat actinogelin; 2, rat skeletal muscle α-actinin; 3, rabbit skeletal muscle heavy meromyosin; 4, chicken gizzard α-actinin; 5, chicken gizzard filamin; 6, chicken gizzard vinculin; 7, rabbit skeletal muscle actin; 8, rabbit skeletal muscle tropomyosin; 9, bovine serum albumin. A marker was spotted on each sheet at upper left corner. Antibody samples used were ascites fluid which were diluted 1000-fold, except for #8-16 which was diluted 100-fold. #24 was treated with 1000-fold diluted nonimmune mouse serum. #25 was not immuno-stained, but was stained for protein with Amido Black to check semiquantitatively the amount of spotted proteins. Number of monoclonal antibodies used for nitrocellulose strips were as follows: 1, chA-I; 2, chA-II; 3, chA-III; 4, chA-IV; 5, chA-V; 6, chA-VI; 7, chA-VII; 8, chα-I; 9, chα-II; 10, chα-III; 11, chA-VIII; 12, chA-IX; 13, chA-X; 14, chA-XI; 15, chA-XII; 16, chA-XIII; 17, chA-XIV; 18, A-II; 19, A-III; 20, A-IV; 21, A-I; 22, A-V; 23, α-I; 26, chA-XV.

Since A-I antibody cross-reacted with rat actiongelin, rat skeletal muscle α-actinin, chicken gizzard α-actinin and filamin, and since all of these proteins bind to the same (or overlapping) binding site on actin (8), it was suspected that this

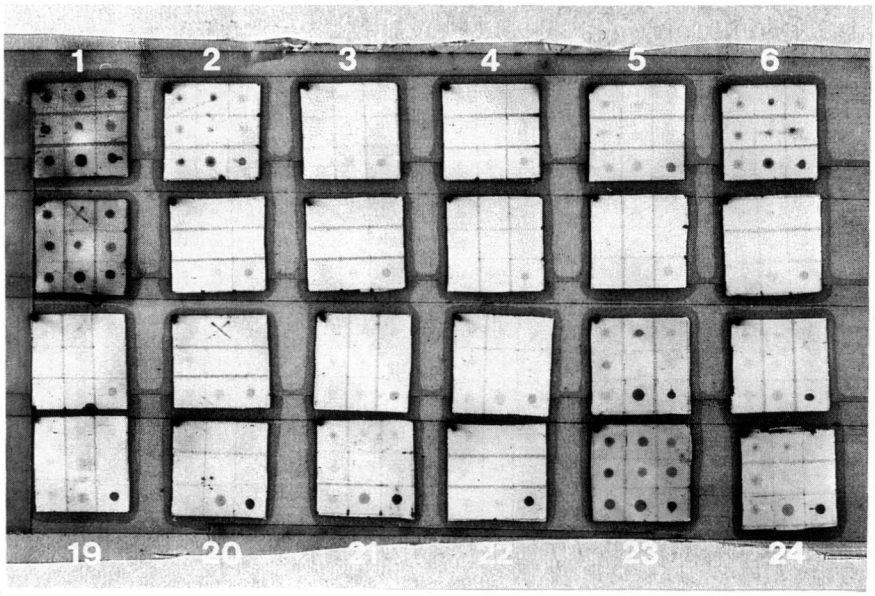

Fig. 2 Dot immunobinding assay of monoclonal antibodies using SDS-denatured antigens. Antigens were boiled in SDS-containing medium as described in "Experimental Procedures", except that #9 grid was spotted with intact chicken gizzard α-actinin. Grid numbers are as described in the legend of Fig. 1, and spotted antigens in these grids were as follows: 1, rat hepatic actinogelin; 2, rat skeletal muscle α-actinin; 3, rabbit skeletal muscle heavy meromyosin; 4, chicken gizzard α-actinin; 5, chicken gizzard filamin; 6, chicken gizzard vinculin; 7, rabbit skeletal muscle actin; 8, chicken hepatic actinogelin; 9, chicken gizzard α-actinin (undenatured). Monoclonal antibodies used for staining and the order of arrangement of the reacted nitrocellulose sheets in the figure were the same as in the legend of Fig. 1, except that α-II was used for #23 and α-I for #24, and except that staining with chA-XV, control serum and Amido Black were not conducted. Grids marked with "X" were not spotted with antigens. Dilution of ascites fluids was 50-fold in all cases.

antibody recognizes an actin-binding fragment of these proteins which may have some similarity in their structure. Thus, a survey on A-I antibody binding site was started using proteolytic fragments of cytoskeletal proteins.

Firstly, one dimensional peptide mapping of cytoskeletal proteins and immunoblotting of the peptide maps were performed (Fig. 3). Electrophoretograms shown in the right-half of the figure are peptide maps which revealed no major similarity among these proteins, although some bands with apparently the same mobility can be found within α-actinin family proteins. By immunoblotting, differences among these proteins became more evident, *i. e.* no identical band(s) even among α-actinin family proteins was observed, except for bands having

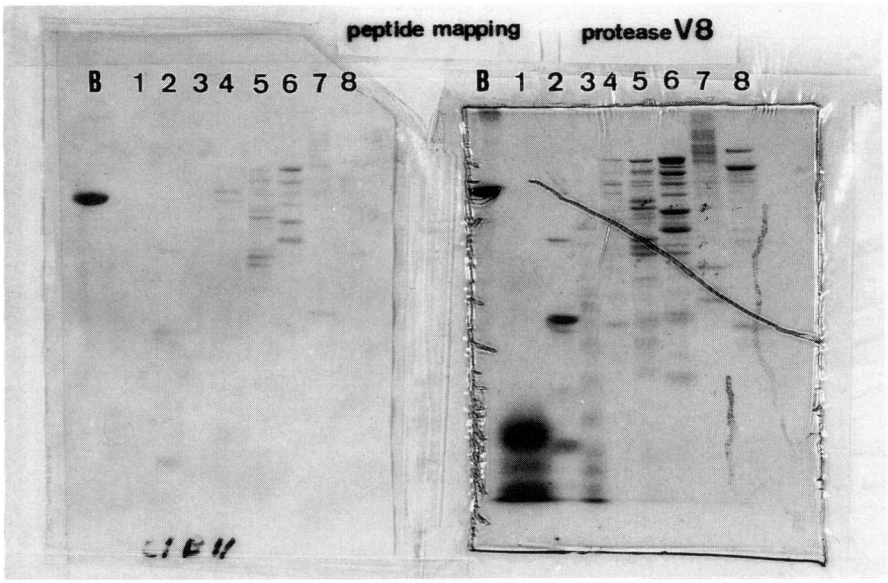

Fig. 3 Immunoblots of V-8 protease peptide maps of various antigens with monoclonal antibody A-I.
The right-half of the figure shows the patterns of staining with Coomassie brilliant blue, and those on the left-half are immunoblotting of the same samples as described in "Experimental Procedures". Antigens that were subjected for peptide mapping using V8 protease were as follows: lane 1, rabbit skeletal muscle tropomyosin; lane 2, rabbit skeletal muscle actin; lane 3, rabbit skeletal muscle heavy meromyosin; lane 4, rat skeletal muscle α-actinin; lane 5, rat hepatic actinogelin; lane 6, chicken gizzard α-actinin; lane 7, chicken gizzard filamin; lane 8, chicken gizzard vinculin; lane B, bovine serum albumin as a molecular mass marker. 12.5% Gel was used.

molecular masses larger than bovine serum albumin (Fig. 3, left-half). Although filamin produced a cross-reacting band of about 30-kD, almost no cross-reacting band was detected with tropomyosin, heavy meromyosin and vinculin. Weak reaction with actin-fragments was noted, and a rather strong cross-reactivity with bovine serum albumin was detected.

Secondly, limited dissection of native proteins with proteases to several domains was tried. As shown in Fig. 4, the α-actinin family proteins were resistant to V8 protease digestion. Chymotryptic and tryptic digests of chicken gizzard α-actinin produced patterns which show some similarity. On the other hand, rat skeletal muscle α-actinin was rather resistant to these proteases. Upon immunoblotting of transfered samples, staining of bands around 30-kD was evident. It should be noted that differences are evident in the staining patterns of chymotryptic digests of these proteins in a higher molecular mass range. This

Fig. 4 Immunoblot using monoclonal antibody A-I of actinogelin and α-actinins limitedly digested with various proteases.
The right-half of the figure shows the staining patterns with Coomassie brilliant blue of antigens which were limitedly digested with V8-protease (lanes, 1-3), chymotrypsin (lanes, 4-6) and trypsin (lanes, 7-9) and subjected to SDS-gel electrophoresis. Patterns on the left-half are the same samples which were transfered to a nitrocellulose paper and immunoblotted with monoclonal antibody A-I. Antigens used were as follows: lanes 1, 4 and 7; rat skeletal muscle α-actinin: lanes 2, 5 and 8; rat hepatic actinogelin: lanes 3, 6 and 9; chicken gizzard α-actinin: lane M; marker proteins consisting of bovine serum albumin (69 kD), actin (42 kD), DNase I (30 kD), trypsin (23 kD) and myoglobin (17 kD). For SDS-PAGE, 12.5% gel was used.

means that some part of these protens are different from each other in accordance with the difference in their sensitivity to Ca^{2+} and in gelation activity, although they belong to the same family.

DISCUSSION

Many monoclonal antibodies against actiongelins and α-actinin were obtained, but none exhibited high binding affinity, since the dilution of ascites fluid up to 1000-fold was the limit for detection of binding. This property is similar to other cytoskeletal proteins, such as actin (10) and tubulin.

It should be noted that many of the prepared antibodies showed different affinities to native and denatured antigens, although some antibodies exhibited reactivity to both native and denatured antigens. This is in accordance with

previous reports (1, 4) indicating that conformation of proteins affects the binding ability of monoclonal antibodies.

Antibodies with the highest specificity so far obtained were those which cross-reacted with three native α-actinin family proteins, *i. e.* rat hepatic actinogelin, rat skeletal muscle and chicken gizzard α-actinins, and denatured chicken hepatic actinogelin. Those with a broader specificity cross-reacted with four α-actinin family proteins and additionally, with chicken gizzard filamin, rabbit skeletal muscle actin or heavy meromyosin. Some of the antibodies cross-reacted with bovine serum albumin, therefore, epitopes which are homologous to that present in the cytoskeletal proteins may be present in this protein.

This finding is not so surprising, since 25 pairs of identical five consecutive animo acid sequences were found in 62 unrelated proteins (2), in other words, about 50% of protein surveyed have one or more such identical sequence(s) which have some possibility of being epitopes. In this survey, only identical sequences were selected, but if such sequences were broadened to allow homologous sequences, the probability of immunological crossreactivity among unrelated proteins may be more than several fold higher. A survey for the presence of identical sequences longer than five residues also appeared recently (15). Furthermore, it is well known that the same or similar epitopes which were detected by the same antibody were sometimes present in unrelated proteins (11, 14).

The nature of the epitope which was detected by antibody A-I was studied further by using limited proteolysis. No common or specific fragments were detected among α-actinin family proteins in peptide maps split with V8 protease. Some specificity was also seen in the filamin sequence, and the 30 kD fragment was rather specifically stained.

Limited digestion of intact molecules with chymotrypsin or trypsin produced a similar and immunostained fragment of about 30 kD between rat hepatic actinogelin and chicken gizzard α-actinin. Since the same antibody also cross-reacted with about 27 kD fragment of thermolytic digests(5), the fragment split off with several protease may have been derived from the same domain. And, since 27 kD domain isolated from thermolysin digests of actinogelin and α-actinins were of the actin-binding domain (5), the fragments described here may also be the actin-binding domain.

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